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Holistic Monitoring of Maine Sea Lice (Lepeoptheirus Salmonis, Kroyer, 1837) Sensitivities to Therapies: Developing a Novel Assay to Examine Lice Behavior

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HOLISTIC MONITORING OF MAINE SEA LICE (*LEPEOPTHEIRUS SALMONIS,* KRØYER, 1837) SENSITIVITIES TO THERAPIES: DEVELOPING A NOVEL ASSAY TO EXAMINE LICE BEHAVIOR

By

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An Abstract of the Thesis Presented
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Sea lice (*Lepeoptheirus salmonis*) present significant economic and animal welfare challenges to salmon aquaculture globally. Chemical delousing agents are used in many countries, with each nation eventually reporting sea lice developing reduced sensitivities to treatments. While some countries have in place sea lice sensitivity monitoring programs, that is not the case in Maine, USA. Although chemical delousing agents are not currently used in Maine, they have been used in the past and are currently used in neighboring Canadian salmon farms. Different bay management areas (BMAs) were sampled during different seasons to determine if there is a seasonal or spatial component to sea lice sensitivities in Maine. Sampling could not be completed for all seasons or BMAs. Using traditional toxicity bioassay methods, lice were exposed to three common chemical delousing agents (emamectin benzoate, hydrogen peroxide, and azamethiphos) to assess their sensitivities to each. It was found that lice in BMA1 had reduced sensitivities to emamectin benzoate. Lice demonstrated sensitivity to azamethiphos. Sea lice initially demonstrated sensitivity to hydrogen peroxide, but after 24 hours post treatment many of the lice had recovered. These variable results highlight the continued need for sea lice sensitivity monitoring in Maine. A monitoring program would help sea lice mitigation strategies on salmon farms.

While traditional toxicity bioassays are useful, they are limited in scope in that they do not consider the sublethal effects of chemical delousing agents on copepodid sea lice. Furthermore, previous methods studying sea lice behavior are typically costly or require extensive equipment setups. A novel behavioral method was developed to assess copepodid behavior in response to exposure to naturally derived compounds. Sea lice behaviors observed using this methodology were similar to sea lice foraging behaviors described in previous work. Contrary to what was demonstrated in previous studies and hypothesized in this thesis, sea lice exposed to isophorone did not exhibit increased overall activity levels or a positive chemotaxis towards the olfactory stimulus. This result suggests that isophorone may play a more complex role in the chemical ecology of salmon farms than previously thought. This highlights the need for further study of the chemical ecology of salmon semiochemicals as it is still poorly understood. The sea lice exposed to putrescine decreased overall activity levels and did not display foraging behavior. This result suggests that putrescine may act as a sea lice repellant and warrants further studies. This novel methodology for studying sea lice behavior is financially and technically accessible to all, and thus may prove to be a reliable way to advance sea lice behavior research in the future.

DEDICATION

To Jalynn M.H. and Caleb Mason—in our hearts and minds always.

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CHAPTER 1

INTRODUCTION

1.1 Salmon aquaculture in Maine

Atlantic salmon (*Salmo salar,* Linnaeus, 1758) farms have existed in Maine since the 1970s, and gradually the face of the industry has changed from small, individually owned farms to larger farms owned by international corporations. Salmon aquaculture is a growing part of Maine's economy, creating stable, sustainable jobs and bringing in $73.4 million in direct output into Maine's economy in 2014 (Cole, Langston, & Davis, 2017). In Maine, the economic impact of the industry nearly tripled between 2007 and 2017, from $50 million to $137 million (Cole, Langston, & Davis, 2017). Salmon aquaculture contributes to providing a high demand seafood product globally, providing a healthy source of protein and Omega-3. However, one of the largest hurdles the salmon aquaculture industry faces are sea lice infestations.

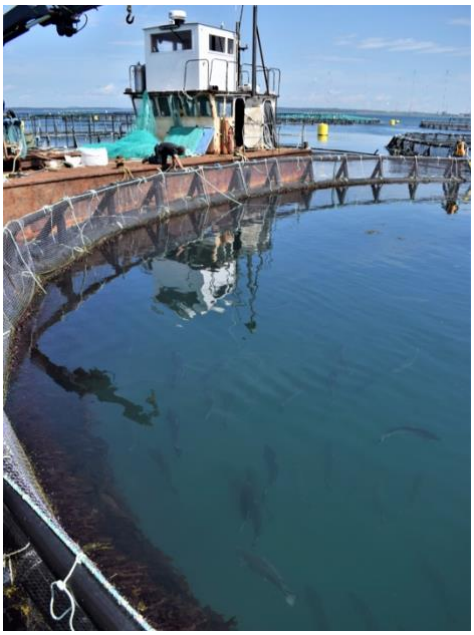

Figure 1.1: A net pen on a typical Maine salmon farm. Photo: Emily

1.2 Sea lice biology

Sea lice, the common name for several marine ectoparasites of the family Caligidae (Order Copepoda: Suborder Siphonostomatoida), are copepods that parasitize fish. There are several species of sea lice that affect salmon aquaculture: in the northern hemisphere primarily *Lepeoptheirus salmonis* (Krøyer, 1837) and *Caligus elongatus* (Nordmann, 1832), and in the southern hemisphere *Caligus rogercresseyi* (Boxshall and Bravo, 2000). In this thesis, the term sea lice refers to species *L. salmonis* only. Starting out life as lecithotrophic free-swimming nauplii, sea lice molt into the copepodid stage, at

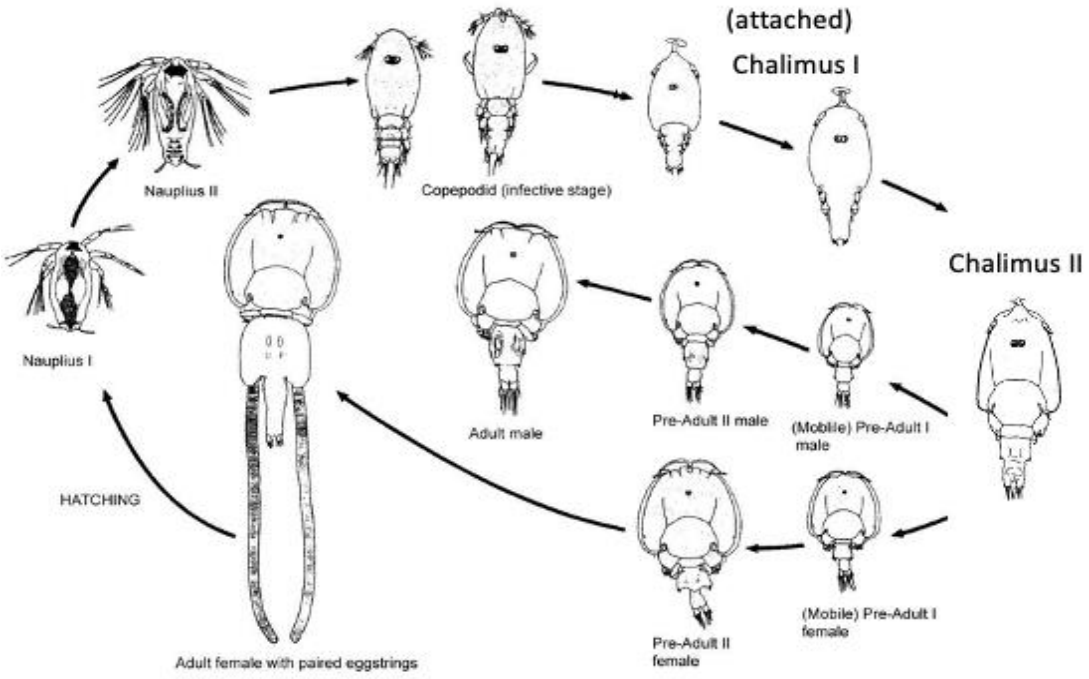

Figure 1.2.1: Life cycle of *L. salmonis*. After Whelan (2010) and Hamre et al. (2013).

which point they can locate and attach to a host (Whelan, 2010; Hamre et al., 2013; Fig. 1.2.1; Fig. 1.2.2). After finding a host, the louse subsequently molts into the chalimus stage, which attaches to the host using a frontal filament produced from a frontal gland (Fast, 2014); the chalimus subsequently molts into the preadult stages, which are mobile (Hamre et al., 2013). Adult lice use the cephalothorax and a modified second antennae to attach to the host. Settled sea lice feed on the mucus, skin, and blood of the host fish; this can cause skin lesions to form, increasing the host's susceptibility to secondary infections (Fast, 2014). In severe lice infestations, the fish can suffer hemodilution and even death (Fast, 2014; Finstad et al., 2000).

Mature adult sea lice will mate with 'virgin' adult females, often with extensive mate-guarding until the deposition of spermatophores (Boxaspen, 2006; Fast, 2014). Despite this, polyandry does occur in sea lice much like other crustaceans (Fast, 2014). A single female louse can produce 6-11 pairs of egg strings throughout its short life of approximately 7 months; the fecundity of the female louse and the viability of the eggs is also variable depending on environmental and host conditions (Boxaspen, 2006;

Fast, 2014). *L. salmonis* can produce 100–1000 eggs/egg string, whereas *C. rogercresseyi* produce only 29 eggs/egg string on average (Mark J. Costello, 2006; Fast, 2014).

The dispersal of planktonic sea lice has been studied extensively (Crosbie et al., 2020; Gillibrandt & Willis, 2007; I. Johnsen et al., 2014; Samsing, Johnsen, et al., 2016; Samsing et al., 2019). Planktonic sea lice can travel tens of kilometers, depending on the oceanography of the region and development times of the lice (Samsing, Oppedal, et al., 2016). This may potentially allow sea lice originating on farms to settle on and harm wild salmonids (M. J. Costello, 2009; Mark J. Costello, 2009c; Whelan, 2010). Furthermore, with the proximity of Canadian salmon farms to U.S. farms, there is a potential for sea lice transfer within the Bay of Fundy.

Figure 1.2.2: *Lepeoptheirus salmonis* copepodid. Scale bar is 100 microns.

1.3 Sea lice management

Not only are sea lice major animal welfare concerns, but they also cause major financial losses to the salmon aquaculture industry. In 2009, global marine salmonid production was 1.7 million tonnes, valued at approximately $8.4billion USD (Mark J. Costello, 2009b). Although a vast body of literature exists on the biology of sea lice in salmon aquaculture, there are very few studies that quantify the economic impacts of sea lice infestations on salmon farms; some of these existing studies utilize voluntary surveys of fish farmers to assess economic impacts (Abolofia et al., 2017; Carpenter, 2019; Mark J. Costello, 2009a; Mustafa et al., 2001; Rae, 2002). Costello (2009a) estimated that sea lice cost

$0.1--$0.2 per kg/fish produced on average globally, which amounts to approximately $360 million, and $1.1 million in Maine, USA alone. Mustafa et al. (2001) estimated that sea lice incur costs on Canadian farms that range anywhere from Can$78,000–$108,000 despite sea lice therapy usage. Abolofia and Wilen (2017) developed a bioeconomic model to calculate the economic costs associated with sea lice on salmon farms in Norway. They found that in 2011, sea lice produced US$436m in damages to the salmon aquaculture industry. However, both of these estimates are likely lower than actual economic costs associated with sea lice infestations.

Sea lice are expensive to treat, and currently, in the state of Maine, the Department of Environmental Protection (DEP) does not allow farms to utilize pharmaceutical treatments used in Canada and elsewhere. Fallowing, hot water and freshwater baths, and provisional licenses for hydrogen peroxide use are the only forms of treatment currently in use in Maine waters. This leaves fish farmers and policy managers with fewer options for sea lice treatment, which may lead to increased resistance to current therapies. Sea lice sensitivities to treatments are assessed using toxicity trials using preadult and adult lice (after Sevetdal and Horsberg, 2003).

The potential of sea lice developing resistance to treatments is a threat to the salmon aquaculture industry (Stian Mørch Aaen et al., 2015). The development of resistance to treatments depends upon less-sensitive individuals surviving until reproduction, thus allowing for microevolution to occur (Kunz & Kemp, 1994). Resistant lice refugia from Canadian salmon farms have the potential to travel to Maine salmon farms and survive until reproduction and pass on resistance genes, exacerbating the issue of decreased sensitivities to delousing agents.

Sea lice can develop resistance to compounds through several mechanisms, such as reduced sensitivities to compounds and poorly applied therapeutants (Stian Mørch Aaen et al., 2015). Subsequently, this has led fish farmers to utilize non-medicinal methods (NMMs) of lice treatment such as fresh water or hot water treatments, snorkel cages, and “cleaner fish” species such as lumpfish

(*Cyclopterus lumpus*) to manage infestations (Groner et al., 2019). Despite the availability of alternative NMM of sea lice treatments, there is some concern regarding decreased sensitivities of sea lice to fresh water treatments (Groner et al., 2019). Parasitic stages of sea lice have shown to be tolerant of waters as low as 7 practical salinity units (PSU) for up to a week while attached to a host salmonid (Groner et al., 2019).

Mechanisms of genetic changes leading to reduced sensitivities to treatments in lice may include point mutations of chemically targeted genes, the upregulation of genes for detoxifying metabolism or efflux pumps in digestion tracks, or other defense mechanisms (Stian Mørch Aaen et al., 2015). The genetic responses to sea lice therapies depends largely on the class of compound used, as pyrethroids, emamectin benzoate, azamethiphos, and hydrogen peroxide utilize different modes of action upon lice systems (as reviewed by Aaen et al., 2015).

Sea lice have demonstrated decreased sensitivities to therapies (Stian Mørch Aaen et al., 2015; Denholm et al., 2002; Helgesen et al., 2015; Marín et al., 2018; Sevatdal & Horsberg, 2003). To assist in combating this issue, other countries have set in place sea lice monitoring programs to track sea lice sensitivity to treatments (Stian Mørch Aaen et al., 2015). Maine does not currently have a sea lice monitoring program, so there is a paucity of continuous spatio-temporal data on sea lice sensitivity. This type of information would be useful in future sea lice mitigation efforts.

Functionally, not much is understood about the ecology of sea lice during the larval stages, particularly the infective copepodid stage. Previous sea lice studies have mostly focused on the adult sea lice biology and host-interactions (Mark J. Costello, 2009a). Some studies have explored sensory cues of copepodids (Stian Mørch Aaen et al., 2015) However, characterizing the behavior of copepodids is essential for understanding their ecological role and infective dynamics at the stage in which sea lice first become infective. By creating a simple, cost-effective, and high-throughput behavioral assay, copepodid behavior can be assessed both in an ecological and an aquaculture pest-management

context. This would be helpful in containing and managing sea lice infestations on salmon farms across the state.

1.4 Research goals

There is not currently any continuous spatio-temporal data on the sensitivity of sea lice to commercial therapeutants in Maine. While there are no authorized chemical treatments currently in use in the state of Maine, the compounds tested in this research have been used in the past in the Gulf of Maine and are still used in neighboring farms in Canada. Furthermore, other nations have established monitoring programs that enable them to stay ahead of sea lice developing resistance to therapies (Stian Mørch Aaen et al., 2015). Maine would benefit from an established monitoring program to track sea lice sensitivity over time; this would assist farmers to develop more functional mitigation strategies for the control of sea lice infestations.

Research objectives were as follows: 1) to determine if there is a seasonal or geographic component to sea lice sensitivities to therapeutants in Maine waters; and 2) to develop a novel behavioral assay for studying sea lice copepodids that is high-throughput and cost-effective. In order to achieve the first research objective, a baseline dataset of lice toxicity assessments across farms and seasons was established. In order to accomplish the second research objective, a behavioral assay was developed and tested with naturally derived compounds. The results of these studies are discussed in the next two chapters.

CHAPTER 2

MONITORING SEA LICE SENSITIVITY TO CHEMICAL THERAPEUTANTS USING TRADITIONAL TOXICITY BIOASSAY METHODS

2.1. Introduction

Salmon aquaculture in the United States has limited options regarding sea lice treatment and management. Currently, there are no approved chemical delousing agents approved for use within the US. There are a couple drugs that are currently in use in Canadian aquaculture such as emamectin benzoate (EMB) and 35% hydrogen peroxide (H_2O_2). However, it may take a considerable amount of time and expense for these treatment options to be deemed safe and effective by the Food and Drug Administration (FDA) for use in US salmon aquaculture. This leaves fish farmers with fewer treatment options; only non-medicinal methods (NMMs) are currently in use such as fallowing, fresh or hot water baths, which have varying degrees of success.

Sea lice have demonstrated decreased sensitivities to several classes of chemical therapies (Stian Mørch Aaen et al., 2015; Denholm et al., 2002; Helgesen et al., 2015; Marín et al., 2018; Sevatdal & Horsberg, 2003), and the potential of sea lice building resistance to treatments is a looming threat to the salmon aquaculture industry (Stian Mørch Aaen et al., 2015). The development of resistance to treatments depends upon less-sensitive individuals surviving until reproduction, thus allowing for microevolution to occur (Kunz & Kemp, 1994). Sea lice can develop resistance to compounds through several ways, such as reduced sensitivities to compounds and poorly applied therapeutants (Stian Mørch Aaen et al., 2015). Some resistance mechanisms to therapeutants have been reviewed thoroughly by Aaen et al. (2015); these may include overexpression of metabolic enzymes and changes in ion channel activity.

Other nations have set in place sea lice monitoring programs to track sea lice sensitivity to treatments (Stian Mørch Aaen et al., 2015). These monitoring programs often utilize traditional toxicity bioassays in which lice are exposed to certain concentrations of test chemicals and assessed for mortality and inactivation (unable to attach to host). Toxicity tests of this nature use a metric known as the effective concentration, or EC_{50}—this is the treatment concentration at which half of the study animals are inactivated by a chemical therapeutant. Similarly, the lethal concentration, or LC_{50}, is the treatment concentration at which half the test subjects expire. These metrics inform policy managers and aquafarmers on best treatment practices and alerts them if local lice populations have a decreased sensitivity to treatments; primarily the EC_{50} is used to measure sea lice sensitivity to treatments (Sevatdal & Horsberg, 2003; Treasurer et al., 2000).

There are few studies examining lice sensitivity to therapies in Maine waters (Gustafson et al., 2006). Maine does not currently have a lice monitoring program, so there is a paucity of continuous spatio-temporal data on sea lice sensitivity to chemical treatments. This type of information would be helpful in controlling and managing sea lice infestations on salmon farms across the state as part of an integrated pest management strategy.

Furthermore, the possibility of sea lice from Canadian farms with exposure to—and potentially lower sensitivities to—common therapies, including emamectin benzoate and hydrogen peroxide, may pose a threat to salmon farms in Maine. The Gulf of Maine is known for large tidal fluctuations, which is able to bring in water from Canadian waters in the Bay of Fundy. Previous studies have indicated that lice are able to travel an upwards of 30km, which creates an opportunity for panmictic populations along the eastern seaboard (Cantrell et al., 2018; Johnsen et al., 2016; as reviewed by Groner et al., 2019). Furthermore, models of lice dispersal have shown lice traveling great distances of tens of kilometers; the modeled dispersal distances varied considerably depending on larval development times and local oceanography (Samsing, Oppedal, et al., 2016). This leaves Maine waters vulnerable to the

transfer of sea lice that are resistant to multiple classes of treatments despite the fact that chemical therapies are not used in Maine salmon aquaculture.

This data chapter aims to create a baseline dataset for sea lice sensitivities to different sea lice treatments used in Canadian salmon aquaculture in order to provide fish farmers and policy managers information on sea lice populations across Maine. There is likely already gene transfer from Canadian lice that have decreased sensitivities with Maine sea lice populations. I hypothesize that Maine sea lice populations will have varying levels of sensitivity to chemical therapies due to geographic location and over time. Additionally, I hypothesize that lice will likely recover from hydrogen peroxide treatments given the nature of lice observed in previous H_2O_2 toxicity studies (Helgesen et al., 2015; Treasurer et al., 2000). In particular, I hypothesize that lice in BMA1 will be the least sensitive out of all the geographic regions for all treatments given its proximity to Canada.

2.2. Methods

Three different bay management areas (BMAs) were designated as different bioregions for sampling efforts (Fig. 2.2.1). Quarterly sampling efforts were undertaken so that there would be at least a full production cycle of salmon followed from smolt to broodstock and harvest. At least two bioassays were run for each BMA sampled in each quarter, farm conditions permitting. Ideally, this would create a baseline database for sea lice sensitivities across a geographical and temporal gradient.

Emamectin benzoate, azamethiphos, and hydrogen peroxide were tested for efficacy of inactivating non-ovigerous adult and pre-adult stage salmon lice *in vitro* using previously established bioassay protocols for each test compound (Bouchard *et al.,* unpubl. after Sevatdal & Horsberg, 2003). While there are no authorized chemical treatments currently in use in the state of Maine, the compounds tested have been used in the past in the Gulf of Maine and are still used in neighboring farms in Canada. Organophosphates have been used in Canada from the mid 1990s–2000 and are

occasionally used as an emergency drug release; hydrogen peroxide is also used sporadically in Canada (Stian Mørch Aaen et al., 2015). Emamectin benzoate has been continually used in Canada since 1999 (Stian Mørch Aaen et al., 2015).

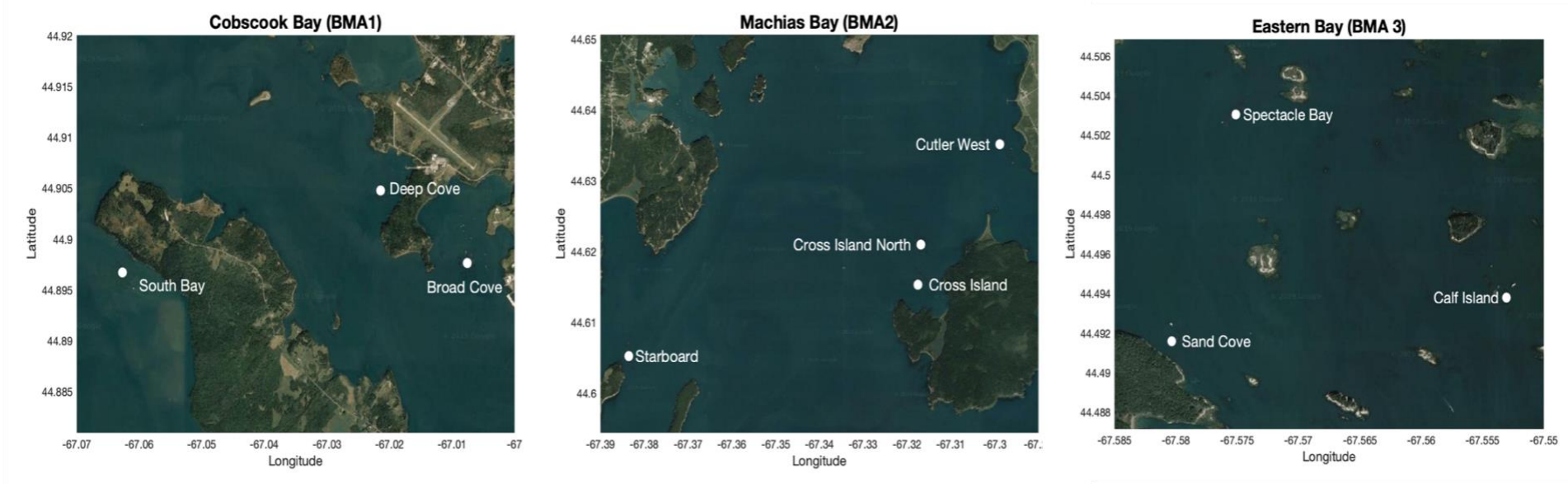

Figure 2.2.1: Sampling sites in each bay management area (BMA) for the state of Maine. Satellite imagery from Google Earth.

Table 2.2.1: Sites sampled in the course of the study.

Quarter	BMA	Site	Compound
Summer 19	1	Broad Cove	H2O2 30min
Summer 19	1	Broad Cove	H2O2 24hr
Summer 19	1	Broad Cove	AZA
Summer 19	1	Broad Cove	EMB
Summer 19	1	Deep Cove	H2O2 30min
Summer 19	1	Deep Cove	H2O2 24hr
Summer 19	1	Deep Cove	AZA
Summer 19	1	Deep Cove	EMB
Summer 19	2	Cross Island	H2O2 30min
Summer 19	2	Cross Island	AZA
Summer 19	2	Cross Island	EMB
Summer 19	2	Cross Island North	H2O2 30min
Fall 19	1	South Bay	H2O2 30min
Fall 19	1	South Bay	H2O2 24hr
Fall 19	1	South Bay	AZA
Fall 19	1	South Bay	EMB
Fall 19	1	Deep Cove	H2O2 30min
Fall 19	1	Deep Cove	H2O2 24hr
Fall 19	1	Deep Cove	EMB
Fall 19	1	Broad Cove	AZA

2.2.1. Hydrogen peroxide bioassay

Salmon lice were collected from market-sized Atlantic salmon from the scheduled sampling sites and acclimated overnight in mesh pots in 30-33$^{0}/_{00}$ recirculating artificial seawater (herein referred to as ASW; Tropic Marin, Wartenberg, Germany) at 12ºC±2. Only lice in good condition (vigorous, attached to side of pot and/or actively swimming) were used for bioassays.

Ten adult and preadult sea lice, five of each sex if available, were randomly sorted into glass petri dishes in triplicate for a total of 30 lice per treatment. Thus, each bioassay had a total of 180 individual sea lice. Ovigerous lice were not used for the current study. Lice were acclimated for 30min in ASW at 12ºC±2. The seawater was then decanted and replaced with geometrically spaced concentrations of hydrogen peroxide (Table 2.1). Lice were exposed to the six different concentrations of hydrogen peroxide (Sigma-Aldrich, St. Louis, MO, USA) for 30min at 12ºC±2 and the treatment water was decanted, and lice were rinsed with ASW. The ASW in the dishes was replaced and mortality assessments were made at 30 min and 24 hours post-treatment (hpt). Multiple mortality assessments were made because lice have been shown to display reduced sensitivities to hydrogen peroxide and even recover after being dosed (Helgesen et al., 2015; Treasurer et al., 2000).

Table 2.2.2: Toxicity bioassay testing concentrations for each sea lice treatment.

Compound	Treatment Concentration (ppm)
Hydrogen peroxide	0
	250
	500
	1000
	2000
	4000
Azamethiphos	0
	0.025
	0.05
	0.1
	0.2
	0.4
Emamectin benzoate	0
	0.2
	0.4
	0.8
	1.6
	3.2

2.2.2. Azamethiphos bioassay

Salmon lice were collected from market-sized Atlantic salmon from the scheduled sampling sites and acclimated overnight in 30-33$^{0}/_{00}$ ASW at 12ºC±2. Only lice in good condition (vigorous, attached to side of pot and/or actively swimming) were used for bioassays.

Ten adult and preadult sea lice, five of each sex if available, were randomly sorted into glass petri dishes in triplicate for a total of n=30 lice per treatment concentration. Thus, each bioassay had a total of 180 individual sea lice. Lice were acclimated for 30min in artificial seawater 30-33$^{0}/_{00}$ ASW at 12ºC±2. The ASW was then decanted and replaced with six concentrations of azamethiphos (Table 2.1). Lice were exposed to the different concentrations of azamethiphos (Sigma-Aldrich, St. Louis, MO, USA) for 1hr at 12ºC±2, and the treatment water was decanted and rinsed off with fresh ASW. The ASW in the dishes was replaced and mortality assessments were made at 24 hours post treatment (hpt).

2.2.3. Emamectin benzoate bioassay

Salmon lice were collected from market-sized Atlantic salmon from the scheduled sampling sites and acclimated overnight in 30-33$^{0}/_{00}$ ASW at 12ºC±2. Only lice in good condition (vigorous, attached to side of pot and/or actively swimming) were used for bioassays.

Ten adult and preadult sea lice, five of each sex if available, were randomly sorted into glass petri dishes in triplicate for a total of n=30 lice per treatment concentration. Thus, each bioassay had a total of 180 individual sea lice. Lice were acclimated for 30min in 30-33$^{0}/_{00}$ ASW at 12ºC±2. The ASW was then decanted and replaced with different concentrations of emamectin benzoate (Table 2.1). Lice were exposed to six different concentrations of emamectin benzoate (Sigma-Aldrich, St. Louis, MO, USA) for 24hr at 12ºC±2, and the treatment water was decanted and rinsed off with fresh seawater. The water in the dishes was replaced and mortality assessments were made at 24hpt.

2.2.4. Evaluation of lice response to therapeutants

Lice were evaluated for overall treatment response using a dissecting microscope at 24hpt (and also 30min post treatment for hydrogen peroxide) as it has been documented that lice EC_{50} values stabilized after 24hpt, but that the mortality of control lice was too high at 48hpt assessments (Sevatdal & Horsberg, 2003). Sea lice were considered dead if they displayed no movement in their extremities, nor in their gut or other internal organs. The lice were considered to be moribund if they could not attach to the side of the dish utilizing the sucking disc or flat body; or if they could not right themselves when flipped over with forceps. Movement of body or organs could still be observed with moribund lice. Lice were considered to be live when they actively suctioned to the sides of the petri dish or swam vigorously around the dish. These lice were able to right themselves when flipped over with forceps, and movement of appendages and/or organs was apparent.

2.2.5. Statistical analysis

The effective concentration at which 50% of the lice were inactivated and the concentration at which 50% of lice died (herein referred to as EC_{50} and LC_{50}, respectively) were assessed for each individual bioassay via probit regression analysis (SPSS Statistics ver. 25.0.0, IBM). In dose-dependent chemical bioassays with arthropods, the probit regression of the percent response is commonly used to assess sensitivity to compounds (J. L. Robertson, 2017). Goodness of fit of the probit models was assessed using a χ^2test. Significance level was set at p=0.05, and 95% confidence intervals were estimated for the individual EC_{50} values. In one evaluation, the collected data fit poorly into a probit regression model which prevented 95% confidence interval estimates.

2.3 Results

Lice treated with AZA had EC_{50} values of 0.089–0.301ppm; Lice treated with EMB had EC_{50} values of 308.7ppm–2208ppm; and lice treated with H_2O_2 had EC_{50} values of 892.2–1687ppm at the 30min assessment timepoint and 1158–2422ppm at the 24hpt assessment timepoint. The recommended dose of hydrogen peroxide for treatment of salmon lice is approximately 1500ppm. While the lice were quick to respond to hydrogen peroxide exposure in 0.5hrs, some lice were able to recover from 'moribund' status entirely at the 24hpt timepoint (Fig. 2.3.1, 2.3.2). In the summer and fall months, lice treated with hydrogen peroxide for 0.5hr had an EC_{50} of ≤1500ppm (Fig. 2.3.1). However, the lice appeared to be less sensitive to hydrogen peroxide at 24hpt, with several of the resulting EC_{50} values exceeding 2000ppm (Fig. 2.3.2).

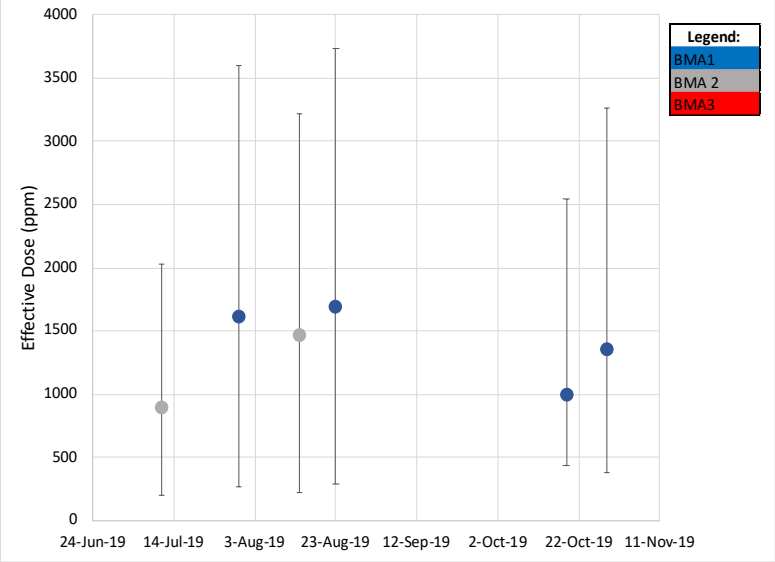

Figure 2.3.1: EC_{50} of hydrogen peroxide after 0.5hpt. Bars represent 95% confidence intervals. Each data point is n=180

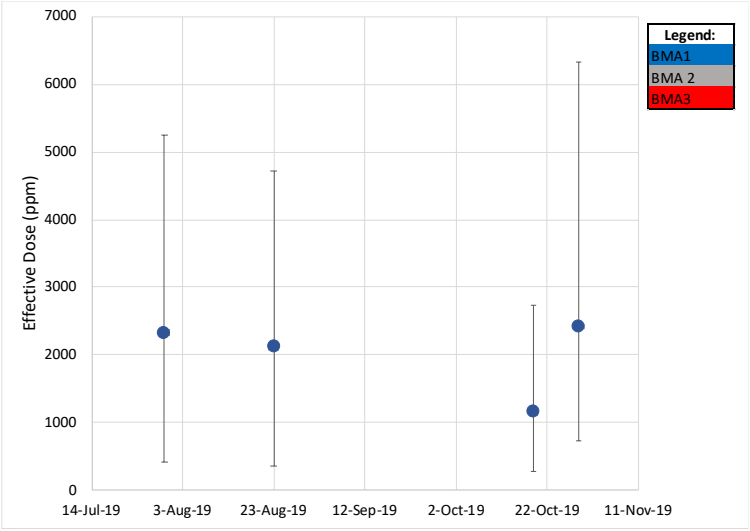

Figure 2.3.2: EC_{50} of hydrogen peroxide after 24hpt. Bars represent 95% confidence intervals. Each data point is n=180

The recommended dose of azamethiphos for treatment of sea lice is 0.1ppm. For the majority of summer and fall bioassays, sea lice had EC_{50} values of approximately 0.1ppm with the exception of the

lice in BMA1 at the summer sampling timepoint (Fig. 2.3.3). The recommended dosage of emamectin benzoate is 50µgkg^{-1}fish^{-1} daily for seven days (in example: approximately 350ppm administered total to a 5kg fish). The EC_{50} values were highly variable, but mostly above the recommended dosages for EMB (Fig. 2.3.4). This may indicate some reduced sensitivity to EMB in BMA 1, the closest location to Canadian farms in the Bay of Fundy. To determine if sea lice are decreasing sensitivities to any one of these compounds tested over time, a longer-term study must be conducted. From the limited amount of data available, it is clear that sea lice sensitivities to compounds is highly variable. Furthering our understanding of sea lice sensitivity to treatments will help inform management decisions in the future.

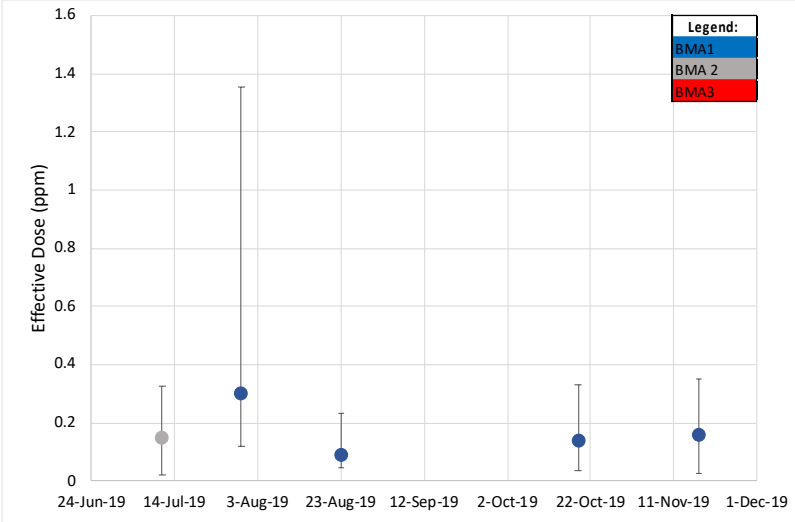

Figure 2.3.3: EC_{50} of azamethiphos after 1hr exposure, assessments made 24hpt. Bars represent 95% confidence

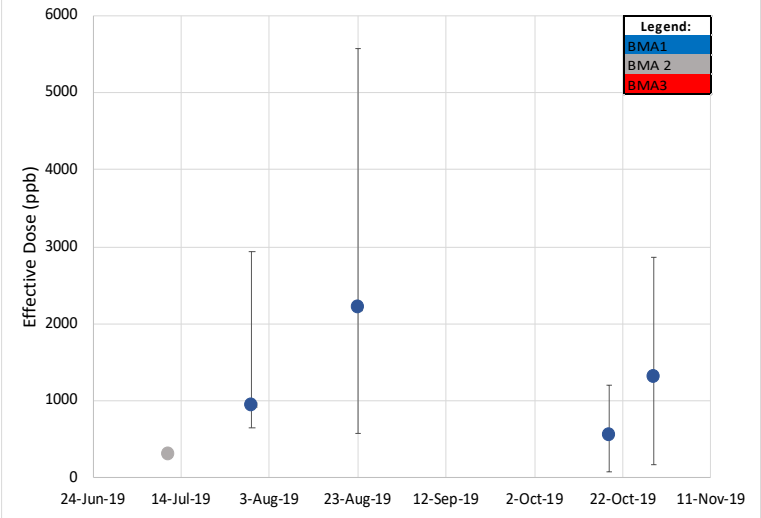

Figure 2.3.4: EC_{50} of emamectin benzoate after 24hr exposure. Bars represent 95% confidence intervals.

2.4. Discussion

The current investigation tested the effects of the administration of three compounds (EMB, AVA, and H_2O_2) on *L. salmonis* to determine sea lice sensitivity to the compounds both seasonally and geographically across Maine. Due to exigent circumstances (COVID-19 pandemic), two seasons of sampling efforts were not able to be completed, thus rendering this dataset incomplete. The results show a variable dose-dependent response to each of the test compounds. Consequently, there is not enough data to make any generalized conclusions regarding the lice populations' sensitivities to chemical therapies in Maine as a whole.

Even with the limited amount of data shown in the present study, there is an alarming lack of sea lice sensitivity to EMB in BMA 1 (Fig. 2.3.4); this is consistent with previous studies' findings regarding EMB (P. Jones et al., 2012; P. G. Jones et al., 2013; Poley et al., 2015; Sutherland et al., 2014; Whyte et al., 2019). While lice responded as expected at the 30min assessment to H_2O_2, a considerable number of lice recovered by the 24hpt assessment, with EC_{50} values up to approximately 1.5x the 30 min assessment values (Fig. 2.3.1; 2.3.2). This is consistent with previous studies on hydrogen peroxide resistance in sea lice (Helgesen et al., 2015; Treasurer et al., 2000). It has been suggested that lice that recover after hydrogen peroxide exposure may be able to reinfect a host salmonid (Treasurer et al., 2000). With the exception of one late summer bioassay, all AZA EC_{50} values were approximately at or below the recommended dosage. The frequency that these compounds are used in Canada may be a factor in the results shown here. As reviewed by Aaen and others (2015), H_2O_2 and organophosphates have only been used sporadically as emergency treatment measures; in contrast, EMB has been used continuously since 1999.

The modes of action for each compound tested is different; this in turn creates the potential for various resistance mechanisms to form in sea lice. The genetic responses to sea lice therapies varies with the class of compound used; pyrethroids, avermectins, organophosphates, and hydrogen peroxide

utilize different modes of action upon lice systems (Stian Mørch Aaen et al., 2015; Chavez-Mardones & Gallardo-Escárate, 2014; Poley et al., 2015; Whyte et al., 2019). EMB targets glutamate-gated chloride channels (GluCl-) by irreversibly forcing them to open (Arena et al., 1995; Cornejo et al., 2014), thereby reducing the cell's excitability (Stian Mørch Aaen et al., 2015). H_2O_2 operates by an etching action and creating gas bubbles in the body, making it difficult for a louse to suction onto the surface of a fish with its cephalothorax; in contrast, AZA targets and inhibits acetylcholine esterases (AChEs) in cholinergic synapses, paralyzing the parasite (as reviewed by Aaen et al., 2015). Mechanisms of genetic changes leading to decreased sensitivities to treatments in lice may include point mutations of chemically targeted genes, the upregulation of genes for detoxifying metabolism or efflux pumps in digestion tracks, or other defense mechanisms (Stian Mørch Aaen et al., 2015; Poley et al., 2015; Whyte et al., 2019).

It is also notable that there is a considerable amount of variance with the EC_{50} values shown. It has been well-documented that arthropods, including sea lice, respond to pesticides differently based on sex (Poley et al., 2015; J. Robertson & Preisler, 1992; Sutherland et al., 2014; Whyte et al., 2019), age, and even size (J. Robertson & Preisler, 1992). This may account for some of the variance shown in the data in the present study. Non-ovigerous adult lice and preadults were selected for use in the study to try to account for that variation in Maine populations in the EC_{50} values. Previous studies (Carmona-Antoñanzas et al., 2016; Denholm et al., 2002; P. Jones et al., 2012; P. G. Jones et al., 2013; Lees et al., 2008a; Sutherland et al., 2014; Whyte et al., 2019) have shown decreases of sea lice sensitivities to chemical therapies with a considerable variance between locations and sampling times. Some of these studies were conducted on lice populations in the Bay of Fundy; moreover, they demonstrate how quickly sea lice lose their sensitivity to chemical therapies. There are few studies of lice sensitivities to chemical therapies in Maine (Gustafson et al., 2006). The results of the present study demonstrate that sea lice sensitivity is highly variable in BMA1 across treatment types. Because of the highly variable

nature of sea lice sensitivities and the proximity of BMA1 to Canadian farms, the need for standardized bioassays monitoring sea lice sensitivities to treatments will be vital in integrated pest management strategies in the future. There still is a lack of understanding on the state of sea lice population sensitivities to chemical therapies and treatment practices across Maine seasonally and geographically.

Current bioassay practices are not necessarily standardized across the field and are often time-consuming and cumbersome (Marín et al., 2018). While toxicant bioassays are optimized for determining dosage effects on lice lethality, they are not designed to look at the sub-lethal effects of chemotherapies on lice behavior, particularly at the copepodid stage. Earlier treatments against sea lice would be ideal in the prevention of sea lice settlement altogether, but currently no behavioral assay exists to meet these research needs. In the next chapter, I present a novel behavioral assay for sea lice copepodids and data from its development.

CHAPTER 3

JUST KEEP SWIMMING: DEVELOPING A NOVEL BEHAVIORAL ASSAY TO EXAMINE SEA LICE COPEPODID BEHAVIOR

3.1. Introduction

Sea lice (*Lepeoptheirus salmonis* Krøyer, 1837) present significant economic and animal welfare concerns in salmon aquaculture (Mark J. Costello, 2009b; Fast, 2014). Traditionally, sea lice outbreaks on farms are mitigated with good husbandry, area management, and chemical delousing agents. However, there are few options for chemical therapies for treating sea lice infestations in Maine, especially with sea lice displaying reduced sensitivities to current therapies elsewhere (Stian M. Aaen et al., 2014; Stian Mørch Aaen et al., 2015; Bravo et al., 2008; Denholm et al., 2002; P. Jones et al., 2012; P. G. Jones et al., 2013; Lees et al., 2008b; Treasurer et al., 2000). Currently available chemical therapies have the potential to negatively impact non-target species and accumulate in the environment as well as the fish treated (Barisic et al., 2019; Cresci et al., 2018; Daoud et al., 2018; Ernst et al., 2014; Gebauer et al., 2017; Samuelsen et al., 2014; Sowles, 2003; Veldhoen et al., 2012).

Sea lice are known to utilize multiple host-related cues to locate and settle upon potential hosts, and in previous studies have been shown to be positively phototactic, particularly with flickering lights designed to mimic fish passing overhead (Fields et al., 2017; Fields et al., 2007; Nuñez-Acuña et al., 2016; Ingvarsdóttir et al., 2013; as reviewed by Mordue (Luntz) & Birkett, 2009a). Sea lice have been shown to display directional responses to host-associated semiochemicals in (Bailey et al., 2006; Ingvarsdóttir et al., 2002; Mordue (Luntz) & Birkett, 2009a; O'Shea et al., 2017). Different sexes and life stages of lice have been shown to increase activity levels in response to host-associated olfactory cues (Bailey et al., 2006; D. Fields et al., 2007; D. M. Fields et al., 2018; Ingvarsdóttir et al., 2002; Mordue (Luntz) & Birkett, 2009b; Núñez-Acuña et al., 2016, 2018, 2019; O'Shea et al., 2017). Another key homing cue for salmon lice is the detection of currents associated with the boundary layer of water

surrounding the salmon body (Mordue (Luntz) & Birkett, 2009a). Salmon lice also have been shown to preferentially settle on different areas of a fish's body at different life stages (Bui et al., 2020).

Traditional bioassay methods focus on examining mortality on preadult and adult sea lice, however, life stages of sea lice respond to stressors differently (as reviewed by Aaen et al., 2015; Jones et al., 2013). Furthermore, traditional bioassay methods are time-consuming and cumbersome, often requiring additional personnel and equipment. This necessitates the development of an assay that can examine sublethal effects of chemical therapies on sea lice biology and behavior.

The use of naturally-derived compounds and kairomones as push or pull compounds in terrestrial agriculture has been well-documented (Mordue (Luntz) & Birkett, 2009a). Compounds derived from garlic and other plants have been shown to repel some terrestrial arthropods as well as decrease activity in sea lice (O'Shea et al., 2017). Fish flesh (including salmon flesh) contains biogenic amines that increase in concentration with decay (Heerthana & Preetha, 2019; Hu et al., 2012; Kamankesh et al., 2019; Laly et al., 2019; Prester, 2011; Wang et al., 2019; Yen & Hsieh, 1991). Biogenic amines have been shown to repel aphids (Sempruch et al., 2016), and have been proposed as a potential repellant for other arthropods. Anecdotally, salmon lice are known to avoid settlement upon dead salmonid hosts.

Previous studies have documented sea lice behaviors such as looping, sinking, and short swimming bursts in response to host-associated cues (Bailey et al., 2006; Devine et al., 2000; D. M. Fields et al., 2018; Mordue & Birkett, 2009; O'Shea et al., 2017). These behaviors are characteristic of an intensive search pattern (Benhamou, 1992; Benhamou & Bovet, 1989). Furthermore, previous studies have demonstrated that sea lice decrease overall activity levels in response to non-host associated cues (Bailey et al., 2006; O'Shea et al., 2017). However, the relationship between the chemical ecology of the natural environment and sea lice behavior still is unclear.

Conventional behavioral assays studying sea lice are costly, require extensive equipment setups, and thus may not be not accessible to all (D. M. Fields et al., 2018; Solvang & Hagemann, 2018). This data chapter aims to create and validate an alternative method for studying sea lice copepodid behavior. In this study, a novel high-throughput behavioral assay was developed in order to examine lice behavior and overall activity levels in the context of a chemical gradient of either push or pull compounds. The biogenic amine putrescine was assessed for its potential as a push compound for sea lice as it is present in decaying salmon flesh. Isophorone, a volatile compound extracted from salmon conditioned water that has been previously shown to increase sea lice activity and positive rheotaxis (Ingvarsdóttir et al., 2002) was assessed as a pull compound in this study. Ultimately, the goal of developing this novel model aims to develop a financially and technically accessible methodology to all. I hypothesize that sea lice will behave differently in response to chemical exposure. Specifically, I hypothesize that lice exposed to isophorone will increase activity levels overall and have a positive chemotaxis toward the source of the stimuli as Ingvarsdóttir and others (2002) have shown in previous studies. I also hypothesize that lice exposed to putrescine will decrease overall activity levels and have a negative chemotactic response.

3.2. Methods

Ovigerous *Lepeoptheirus salmonis* were collected from market-sized Atlantic salmon in net pens on a salmon farm in Machiasport, Maine, USA. Egg strings were carefully removed from the female lice with forceps and incubated in 33ppt Tropic Marin artificial seawater (herein referred to as ASW; Tropic Marin, Wartenberg, Germany) at 10°C ± 2 in a small recirculating system. As egg strings hatched, nauplii were sorted into different cohorts by hatch date into PVC containers outfitted with 150µm mesh. Lice were reared until the five-day-old copepodid stage, at which point they were used in behavioral assays. Lice stage and condition were determined with a stereomicroscope.

3.2.1. Videography setup

Four custom-made light boxes (30.48cm X 25.4cm X 30.48cm each) inside a custom-made wooden frame were illuminated with two 60w LED bulbs, approximating 800 lumens per bulb (Fig 3.2.1). The incident angle of light into the lightboxes was approximately 45º such that shadows from the camera mount were minimized. All videos were captured with mounted Panasonic HC-WXF991 camcorders set at 4k high-definition settings at a 60fps frame rate.

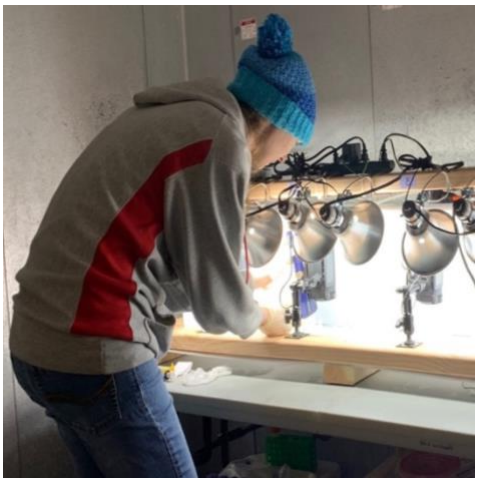

Figure 3.2.1: Lightbox setup in environmental chamber. Photo courtesy of M. Scarlett Tudor.

3.2.2. Characterizing lice behavioral responses to chemical gradient

Custom-made glass rectangular dishes (8.9cm X 55cm) were filled with 30mL artificial seawater (herein referred to as ASW) and held constant at 10ºC ± 2 in an environmental chamber. The dish was divided into four quadrants in order to assess time spent in different sections of the arena. Putrescine dihydrochloride (Sigma-Aldrich, St. Louis, MO), alpha-isophorone (98% v/v; Sigma-Aldrich, St. Louis, MO), or ASW were added to the side of the dish for final treatment concentrations of 20ppm (putrescine) and 200ppb (isophorone). Isophorone has been identified in and isolated from salmon-conditioned water and has been shown to elicit a positively taxic response in sea lice in a previous study (Ingvarsdóttir et al., 2002). These target concentrations were selected based on the desired concentration after the compound had completely diffused across the dish; these calculations were based off a theoretical diffusion gradient model (WL Gore & Associates, unpubl.). The putrescine concentration was based on what putrescine concentrations previous studies observed in fish flesh during the decay process (Heerthana & Preetha, 2019; Hu et al., 2012; Kamankesh et al., 2019; Laly et al., 2019; Prester, 2011; Wang et al., 2019; Yen & Hsieh, 1991).

In a preliminary behavioral assay, various isophorone concentrations were examined to verify the positive taxis effect Ingvarsdóttir and others observed (see Appendix C for results). The compound was added randomly at either the left or the right side of the dish in order to create a diffusion gradient. The louse was added to the center of the arena and filmed for a total of five minutes. Each compound was tested in each lightbox and on both sides of the dish to minimize potential side biases of the copepodids.

3.2.3. Statistical analysis

Videos were binarized in MATLAB R2019a (The Mathworks Inc, USA) prior to processing for trajectory data using idTracker software (Pérez-Escudero et al., 2014). This software developed by Pérez-Escudero and others has been used and validated for tracking zebrafish, mice, and insects; idTracker is also open-access software. Tracking data were further processed using MATLAB using custom-written codes developed by the author for use with this methodology. See Appendix 1 for custom-written MATLAB codes. Videos in which sea lice could not be reliably detected in idTracker were excluded from the analysis.

The residence time in arena quadrants, total distance traveled, and velocity was calculated for each louse video. In residence time analyses, quadrant 1 (Q1) was considered to be the quadrant in which the compound was added, regardless of whether it was the left or the right. Similarly, quadrant 4 (Q4) is the furthest area from where the treatment compound was added, regardless of whether the compound was added on the left or right side of the dish. Additionally, the tortuosity entropy (herein referred to as TorEn) was calculated for each video (Liu et al., 2015). A principal components analysis was conducted on the previously described behavioral metrics in JMP statistical software (v. 15.2.0; SAS Institute Inc.).

Data were subjected to Levene's tests for homogeneity of variance; $P_L < 0.05$ indicates heteroscedasticity. Data for isophorone and control treatments from different sampling time points were tested for differences with a student's t–test and found to not differ statistically ($P > 0.05$), therefore these data were pooled. Differences in velocity, distance traveled, residence time, and TorEn were compared among treatments with Welch's ANOVAs with log-transformed data; $P < 0.05$ indicates statistical significance. All following graphs are shown with the original data.

3.3. Results

The tracking software idTracker was able to detect sea louse copepodid movements with variable results. While the tracking did sometimes have noise, this issue was generally remedied by adjusting the tracking parameters in the graphic user interface of idTracker. The translucent nature of the copepodid cephalothorax made tracking challenging with suboptimal lighting conditions. However, once lighting conditions were optimized, idTracker was able to detect the lice reliably. Qualitatively, several behavioral patterns were observed in many subjects, such as horizontal 'looping' and quick accelerations or 'swimming bursts' (as exemplified in Fig. 3.3.1). See Appendix B for all custom-written codes used for analyzing idTracker data.

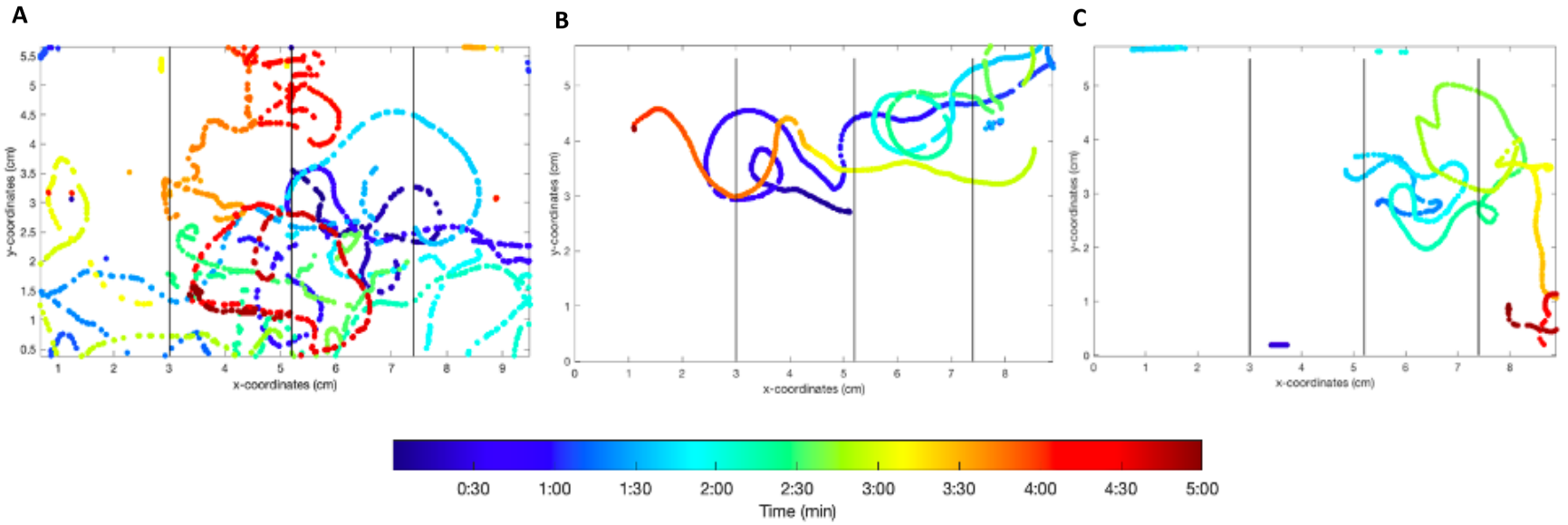

Figure 3.3.1: Sample sea lice track output from idTracker software. Each video assessment is a five-minute duration. Vertical lines indicate quadrant boundaries. Panel A is seawater control, Panel B is the 200ppb isophorone treatment, Panel C is 20ppm putrescine.

A principal components analysis was conducted to determine the structure of the dataset and detect any patterns in biological endpoints measured. Total distance traveled accounted for the vast majority of the variance observed in the dataset (95%; Figure 3.3.2). Residence time in Q1 accounted for a small percentage of the variation observed (3.22%; Figure 3.3.2).

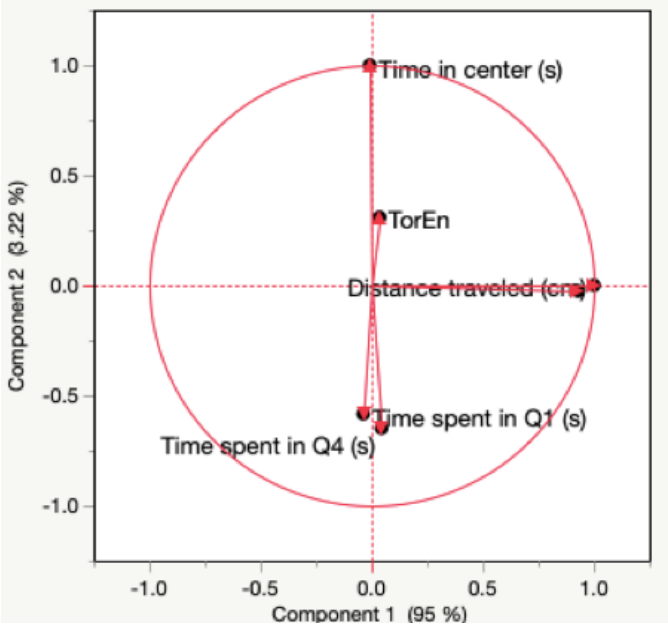

Figure 3.3.2: Principal components analysis of behavioral endpoints measured in sea lice. The variable in question is labeled next to the eigenvector arrows. PC1 accounts for 95% of the variation, and PC2 accounts for 3.22% of the variation in the dataset.

The lice treated with isophorone or putrescine traveled less than control group lice, though this effect was not statistically significant (Figure 3.3.3; $P > 0.05$). The chemical diffusion gradient in the assay was implemented to assess if there was a directionality to the

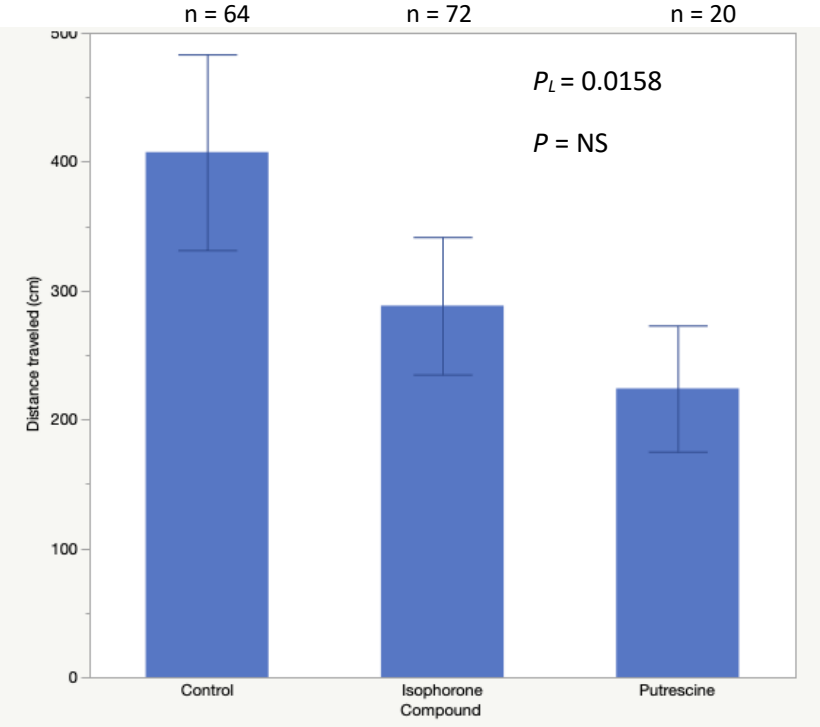

Figure 3.3.3: Total distance traveled by copepodids. Vertical error bars represent standard error of the mean. P_L refers to the Levene's test result; a significant amount of heteroscedasticity is considered a value of $P_L<0.05$.

louse movements, and if there was a repellant or attractant effect to each compound tested. However, in each treatment group, the lice spent the most time in the center of the dish compared to either side of the dish, regardless of whether the compound was added in that quadrant or not (Figure 3.3.4). Contrary to what was hypothesized, lice treated with putrescine spent significantly more time in Q1 than control lice (Figure 3.3.4; $P = 0.0173$).

There was a trend for sea lice treated with putrescine to travel slower than control lice, though this result was also not statistically significant (Figure 3.3.5, $P > 0.05$). Although not statistically significant, lice treated with isophorone also traveled slower than control lice (Figure 3.3.5). The TorEn

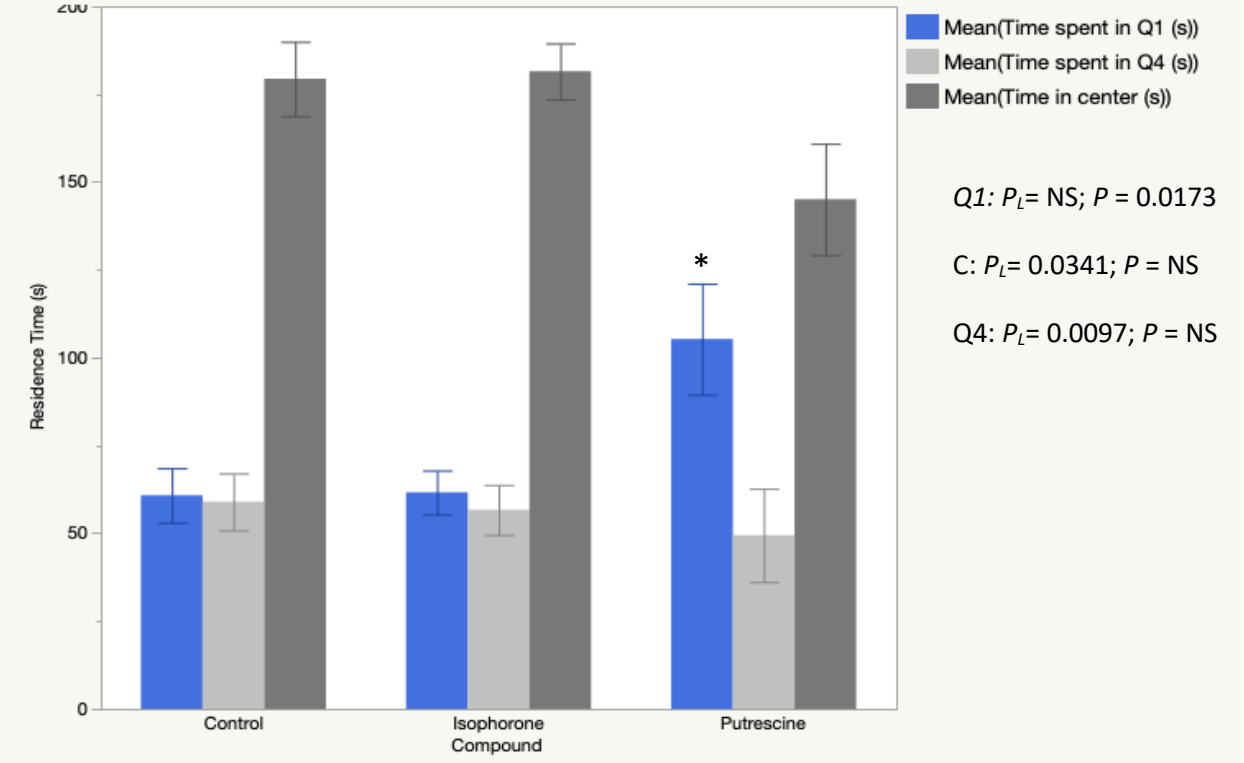

Figure 3.3.4: Time copepodids spent in each arena quadrant. Vertical bars represent standard error of the mean. Quadrant 1 (Q1) is always the side in which the compound was added, whereas Q4 is the furthest from where the compound was added. P_L refers to the Levene's test result; a significant amount of heteroscedasticity is considered a value of $P_L<0.05$.

of lice treated with putrescine was significantly lower than the controls and lice treated with isophorone (Figure 3.3.6; $P = 0.0448$).

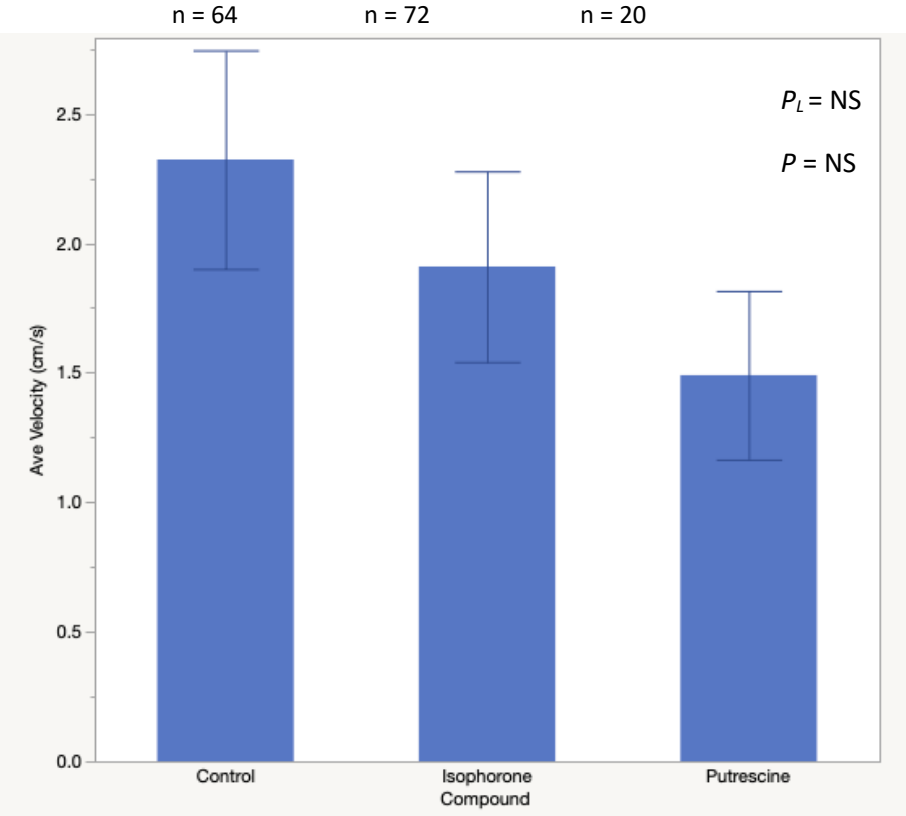

Figure 3.3.5: Average velocity of lice by treatment group. Vertical bars represent standard error of the mean. P_L refers to the Levene's test result; a significant amount of heteroscedasticity is considered a value of $P_L<0.05$.

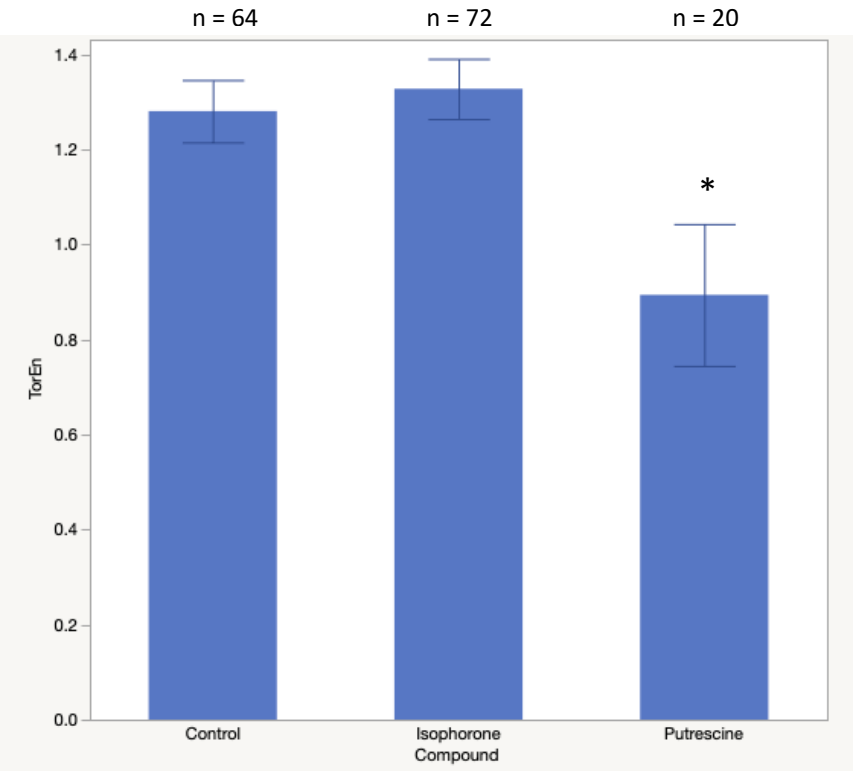

Figure 3.3.6: Mean TorEn of lice by treatment group. Vertical bars represent standard error of the mean. P_L refers to the Levene's test result; a significant amount of heteroscedasticity is considered a value of $P_L<0.05$. $P_L = 0.0009$; $P = 0.0448$

3.4. Discussion

The present study developed a novel, accessible method for studying sea lice copepodid behavior. This model was tested in the context of an artificially produced chemical gradient with a known lice attractant derived from salmon-conditioned water and a naturally derived terrestrial arthropod repellant. The sea lice behaviors observed in this study such as looping and swimming bursts are consistent with behaviors observed in previous studies (Figure 3.3.1; Bailey et al., 2006; Devine et al., 2000; D. M. Fields et al., 2018; Ingvarsdóttir et al., 2002; Mordue (Luntz) & Birkett, 2009a; O'Shea et al., 2017). As the depth of the arena minimized vertical movement, sinking behaviors were not quantified in the tracking data (only two-dimensional movement was quantified).

It is known that sea lice respond to a variety of physical stimuli that increases activity level or induces behavior associated with attaching to a host; these may be olfactory cues, visual cues such as a change in light, or a mechanical cue such as water currents (Bailey et al., 2006; Browman et al., 2004; D. Fields et al., 2007; D. M. Fields et al., 2018; Flamarique et al., 2000; Ingvarsdóttir et al., 2002; Mordue & Birkett, 2009; Núñez-Acuña et al., 2018, 2019). The experimental design had a constant light intensity and no moving water currents to minimize the effects of these stimuli on sea lice behavior. Ingvarsdóttir et al. (2002) identified the compounds α-isophorone and 1-octen-3-ol as semiochemicals within salmon conditioned water that attracted sea lice and increased their overall activity. Only isophorone was used in the present study. The lice treated with isophorone or putrescine covered less distance overall, however, this effect was not statistically significant (Fig. 3.3.3). However, it is worth noting that the variances decreased despite the smaller sample size for lice treated with putrescine. While statistical significance of results and other means of central tendency are helpful and informative, animal behavior is not always so apparent statistically. It is also known that the variances and distributions of data change with regards to animal physiology in response to environmental contaminants; as such, it has been proposed to utilize the change in variances or distributions as another means to assess differences in physiological responses to contaminants (Orlando & Guillette, 2001).

With that in mind, most of the data in the present study (with the exception of velocity data and residence time in Q1) displayed significant heteroscedasticity. Much of the heterogeneity shown in the variances was in the putrescine group compared to control groups (see Figs. 3.3.3–3.3.6). Because of the large number of variables and the number of behavioral metrics applied in this study, a PCA was conducted to determine the structure of the dataset and detect any potential patterns in the biological endpoints measured (Fig. 3.3.2). Most of the variance shown in the results of this study can be attributed to the total distance traveled by lice (PC1 = 95%, Fig. 3.3.2). The residence time in Q1 accounted for the majority of the remainder of the variation observed (PC2 = 3.22%, Fig. 3.3.2). This

result, along with the large degree of heteroscedasticity in the distance data, may indicate that the metric of total distance traveled is an important component in overall activity levels of sea lice, despite the lack of statistically significant means.

Sea lice exposed to putrescine had a significantly higher residence time in Q1 compared to the control, contrary to what was hypothesized *a priori* to the experiment (Fig. 3.3.4). Additionally, the residence time variances displayed significant heteroscedasticity compared to the control; the lice treated with putrescine tended to have higher variances. Salmon lice exposed to isophorone did not differ significantly in residence time from the control, which was also the opposite effect to what was hypothesized. Ingvarsdóttir et al. (2002) have previously shown that isophorone (a component in salmon conditioned water) causes positive chemotaxis in sea lice as well as increases their overall activity levels (i.e. distance traveled, velocity). Similarly, it has been demonstrated that putrescine and other biogenic amines act as a repellant to some terrestrial arthropods (Sempruch et al., 2016). Previous studies examining sea lice behavior often utilized Y-tube olfactometers to assess behavioral choices made by sea lice (Bailey et al., 2006; Devine et al., 2000; O'Shea et al., 2017).

Lice that spent the majority of the time in the center of the arena were categorized as not making directional choices for the purpose of this study; however, the interaction of the lice with the chemical environment of the test arenas is likely more complex than a simple choice or no-choice test. The present study employed the use of a chemical gradient that minimized water currents that may invoke sea lice activity. The assessment length of five minutes was determined by the time it took test compounds to reach the center of the dish in theoretical diffusion models (WL Gore & Associates, unpublished data). However, these diffusion models must still be validated in order to optimize the model.

Sea lice exposed to isophorone and putrescine had lower average velocities than the control, however this effect was not statistically significant. Sea lice exposed to putrescine had a significantly

lower TorEn compared to the control, whereas lice exposed to isophorone did not differ from the control lice (Fig. 3.3.6). As reviewed by Mordue-Luntz and Birkett (2009), sea lice have shown to increase their swimming speed and activate foraging behavior (i.e. looping, sinking and swimming intensive search patterns) in response to detecting host cues, and decrease in activity levels in response to non-host associated chemicals. The positive chemotaxis and increased activity levels displayed by sea lice in previous studies in response to isophorone was not observed in the present study (Fig. 3.3.1-3.3.6; see supplementary data in Appendix C). Even more noteworthy was that this result was not observed in Y-tube assays; the results of those assays appeared to have the opposite effect (Morefield, unpublished data). This, in concordance with Morefield's results, suggests that isophorone may have a more complex role in the chemical ecology of marine ecosystems than previously thought. While there are a few compounds identified in salmon conditioned water, there is still much to learn about the nature of the olfactory cues released by salmonid hosts. Mordue-Luntz and Birkett (2009) have suggested that some of the previously described compounds isolated from salmon conditioned water may not be attractants, but rather should be classified as phagostimulants that cue salmon lice to feed once landed on a potential host.

The sea lice exposed to putrescine had significantly fewer complex movements (Figs. 3.3.1 and 3.3.6) and in general had lower activity levels (Figs. 3.3.3 and 3.3.6). This suggests that putrescine may deter lice at concentrations of 20ppm. Future studies should examine putrescine as a potential repellant at different concentrations. There may be another concentration of putrescine that consistently repels sea lice from salmon; however, the range of detection of putrescine by sea lice is not currently known. Sea lice were also exposed to cadaverine, another biogenic amine associated with fish decay, but these results were not included in the study because the cadaverine reacted with seawater to create a solute.

In conclusion, the behavior methodology presented here is a valuable new tool for researchers and policy managers alike. The materials and associated data processing software are inexpensive and,

in some cases, free. The unique capabilities of idTracker software allowed multiple behavioral endpoints to be measured. Liu and others (2015) developed TorEn as a robust index for measuring path complexity of animals. This index builds off of the sinuosity index and other random walk behavioral metrics developed previously (Benhamou, 1992, 2004a, 2004b, 2006; Benhamou & Bovet, 1989). This study is the first to apply the TorEn index to sea lice behavior. The TorEn proved to be an effective method for quantifying the path complexity of sea lice copepodids. In this study, lice did not significantly increase activity levels or path complexity in response to isophorone exposure as demonstrated in aforementioned studies. This, in concordance with Y-tube olfactometer data (Morefield, unpublished data), indicates that isophorone may have a more complex role in the environmental chemical ecology and sea lice host location. Finally, this study demonstrated the potential for putrescine as a sea lice copepodid deterrent. Future studies should examine the effects of different concentrations on lice behavior to determine the most effective dosage.

CHAPTER 4

CONCLUSIONS

This thesis examined the sensitivities of sea lice preadults and adults to three chemical delousing agents commonly used in salmon aquaculture. While the goal was to sample across the span of at least a year at three different BMAs, this was not possible due to exigent circumstances. However, the obtained results of this study suggest that sea lice in BMA1 have reduced sensitivities to emamectin benzoate. Lice in this region still displayed susceptibility to azamethiphos. While lice initially were inactivated by hydrogen peroxide, by the 24hpt observation, most lice had recovered as previous studies have described. These results highlight the need for a continued sea lice sensitivity monitoring program in Maine, USA to help with salmon farm management and sea lice mitigation efforts.

In this thesis, a novel, high-throughput approach to studying sea lice copepodid behavior was developed. Sea lice behaviors observed using this methodology were similar to those described in previous work. The sea lice exposed to isophorone did not exhibit increased overall activity levels or positive chemotaxis as previous studies have described. These results suggest that isophorone is not a simple pull compound but might instead be a phagostimulant for sea lice. This highlights the need for additional studies of the chemical ecology of salmon semiochemicals as it is still poorly understood. The sea lice exposed to putrescine decreased overall activity levels and did not display foraging behavior. This result suggests that putrescine may act as a sea lice repellant and warrants future studies. This novel methodology for studying sea lice behavior is financially and technically accessible to all, and thus may prove to be a reliable way to advance sea lice behavior research in the future.

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APPENDIX A: SUPPLEMENTARY TOXICITY BIOASSAY DOSE RESPONSE GRAPHS

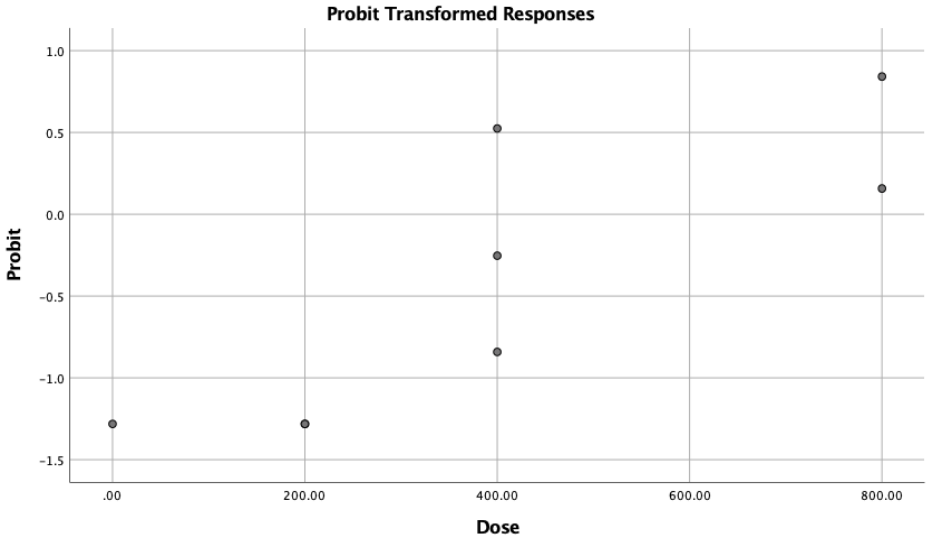

Figure A.1: Example of a probit regression of dose-dependent response of sea lice treated with emamectin benzoate. This type of regression is estimated in SPSS statistical software to determine the EC_{50} of each toxicity bioassay conducted.

APPENDIX B: MATLAB CODES

```
%This is combining batch_distance and batch_time_quadrants codes for
%getting both variables at once from trajectory data
%%
%%For calculating total distance traveled by sea louse
for i= 1:76 %video numbers, change as needed
   FileBase = ['/Volumes/SeaLiceVideos4/6June19collection/' num2str(i) '/']; %file directory
   trajectoryFileName = [FileBase, 'trajectories.mat']; %trajectory file
   if exist(trajectoryFileName, 'file') %checks if file exists before proceeding
      %file exists, do all this stuff...
      load(trajectoryFileName); %change directory as needed
      X=trajectories(1:end,:,1); %change range of variables as necessary to analyze different frame
number range (Originally was a ':' in the first argument)
      Y=trajectories(1:end,:,2); %1:9000 is approximately the first 5 minutes of video, use 9000:end to
look at second half separately
      X_cm=X/362; % conversion ratio for a 4K resolution for Panasonic HC-WXF991 camcorder (zoomed
in to fit dish in screen)
      Y_cm=Y/362;
      for j=1:size(X_cm)-1
         k(j)=sqrt(((X_cm(j+1,1)-X_cm(j,1))^2)+((Y_cm(j+1,1)-Y_cm(j,1))^2)); %distance formula, calculates
distance between each point
      end
      distance_total(i)=nansum(k); %adds all distances

   else
      %file does not exist...give warning message
      warningMessage = fprintf('Warning: file does not exist:\n%s', trajectoryFileName);
%       uiwait(msgbox(warningMessage));
   end
end
%% save distances for posterity
% save('/Volumes/SeaLiceVideos2/5Dec18collection/batch_total_distance', 'distance_total'

%% Input variables for batch_time_quadrants
numVideos = 76; % input("Number of videos: ");
includeInterpolation = 0;

% for calculating time louse spent in each quadrant in arena
quadrantTime = zeros(numVideos, 4);
for i= 1:numVideos %video numbers, change as needed
   FileBase = ['/Volumes/SeaLiceVideos4/6June19collection/' num2str(i) '/']; %file directory
   trajectoryFileName = [FileBase, 'trajectories.mat']; %trajectory file
   if exist(trajectoryFileName, 'file') %checks if file exists before proceeding
      %file exists, do all this stuff...
      load(trajectoryFileName);
```

```
X=trajectories(:,:,1); %change range of variables as necessary to analyze different frame number range (Originally was a ':' in the first argument)
Y=trajectories(:,:,2); %1:9000 is approximately the first 5 minutes of video, use 9000:end to look at second half separately
X_cm = X/362; %pixel to cm conversion ratio = coordinate/362
Y_cm = Y/362; %pixel to cm conversion ratio = coordinate/362
%% Defining quadrantSize and the correction factors for FOV
z = 0.8; %correction factor for distance (cm) between side of dish and edges of video
quadrantSize = 2.2; % the size of each quadrant
%% Calculating time spent in quadrant
k = 1;  % declaring variable for loop iteration
currentQuadrant = ceil((X_cm(k) - z) / quadrantSize); % get the starting point
while (isnan(currentQuadrant)) % if the starting point is NaN, continue untli we find a good one
   k = k + 1;
   currentQuadrant = ceil((X_cm(k) - z) / quadrantSize);
end
if (currentQuadrant > 4)    % coercing initial value to 1 through 4
   currentQuadrant = 4;
end
if (currentQuadrant < 1)
   currentQuadrant = 1;
end
nextQuadrant = currentQuadrant; % Declaring variable for loop iteration
sizeX = size(X_cm, 1);  % the number of coordinates
for j = k : sizeX

   if (j < sizeX && ~isnan(X_cm(j + 1))) % if we have another point after this one...
      nextQuadrant = ceil((X_cm(j + 1) - z) / quadrantSize);

      % coercing to the bounds [1,4]
      if (nextQuadrant > 4)
         nextQuadrant = 4;
      end
      if (nextQuadrant < 1)
         nextQuadrant = 1;
      end
   end

   % Checking interpolation
   if (includeInterpolation == 1 && currentQuadrant ~= nextQuadrant && ~isnan(X_cm(j + 1)) && ~isnan(X_cm(j)))
      %        border = (nextQuadrant * 2.2) + z;
      %        pre = .5;
      %        post = .5;
      %        if (X_cm(j + 1) > X_cm(j))
      %           distance = X_cm(j + 1) - X_cm(j);
      %           pre = (border - X_cm(j)) / distance;
      %           post = (X_cm(j + 1) - border) / distance;
```

```
            %           else
            %               distance = X_cm(j) - X_cm(j + 1);
            %               pre = (border - X_cm(j + 1)) / distance;
            %               post = (X_cm(j) - border) / distance;
            %
            %           end
            %           quadrantTime(1, currentQuadrant) = quadrantTime(1, currentQuadrant) + ((1/30) *
pre);
            %           quadrantTime(1, nextQuadrant) = quadrantTime(1, nextQuadrant) + ((1/30) * post);

            % Adding it to the quadrant
          else
            quadrantTime(i, currentQuadrant) = quadrantTime(i, currentQuadrant) + 1/30;
          end

          % moving to next point
          currentQuadrant = nextQuadrant;
        end
      else
        %file does not exist...give warning message
        warningMessage = fprintf('Warning: file does not exist:\n%s', trajectoryFileName);
      end
    end
    %% save distances for posterity
    % save('/Volumes/SeaLiceVideos2/5Dec18collection/quadrantTime', 'quadrantTime')
    %% Code is finished running!! Ask user if you want to quit MATLAB??
    beep on
    beep
    beep off
    message = 'Program finished running, would you like to quit MATLAB?'; %display message box
    reply = questdlg(message, 'Quit MATLAB', 'OK', 'Cancel', 'OK'); %gives user options to reply
    if strcmpi(reply, 'OK')
      quit; %user said Quit, so exit MATLAB.
    end
```

APPENDIX C: ISOPHORONE PRELIMINARY TRIAL RESULTS

Different concentrations of isophorone were used to attempt to determine an optimal concentration for attracting lice; others have used isophorone as a 'positive control' or attractant in previous studies (Ingvarsdóttir et al., 2002). Copepodids exposed to 200ppb isophorone and 200pptr isophorone did not have significant differences in total distance traveled than copepodids in ASW. In contrast, sea lice exposed to 200ppq isophorone traveled a significantly lesser distance than copepodids in ASW (P=0.0249; Fig. C.1). There were no significant differences between the amount of time spent in quadrants 1 and 4; furthermore, most of the time spent by copepodids was in the center of the arena (Fig. C.2).

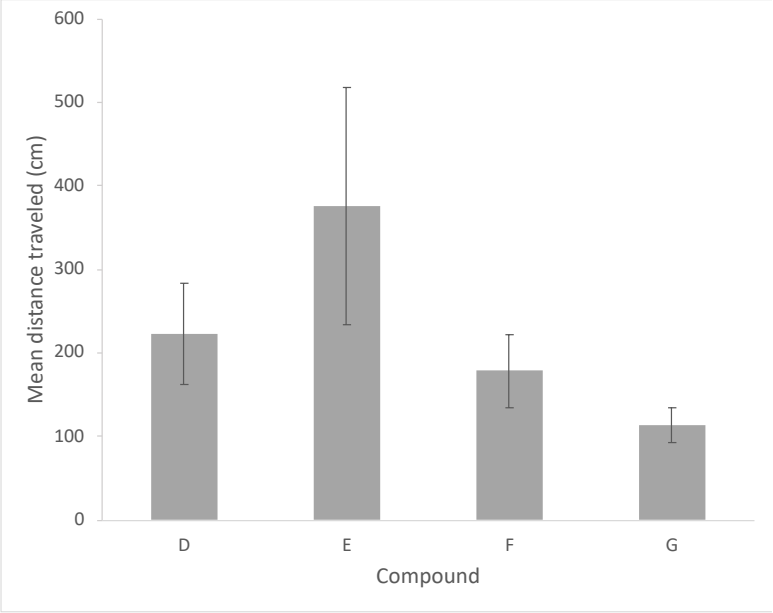

Figure C.1: Total distance traveled by lice. Vertical bars represent standard error about the means. Treatments: D = 200pptr isophorone; E = ASW; F = 200ppb isophorone; G = 200ppq isophorone.

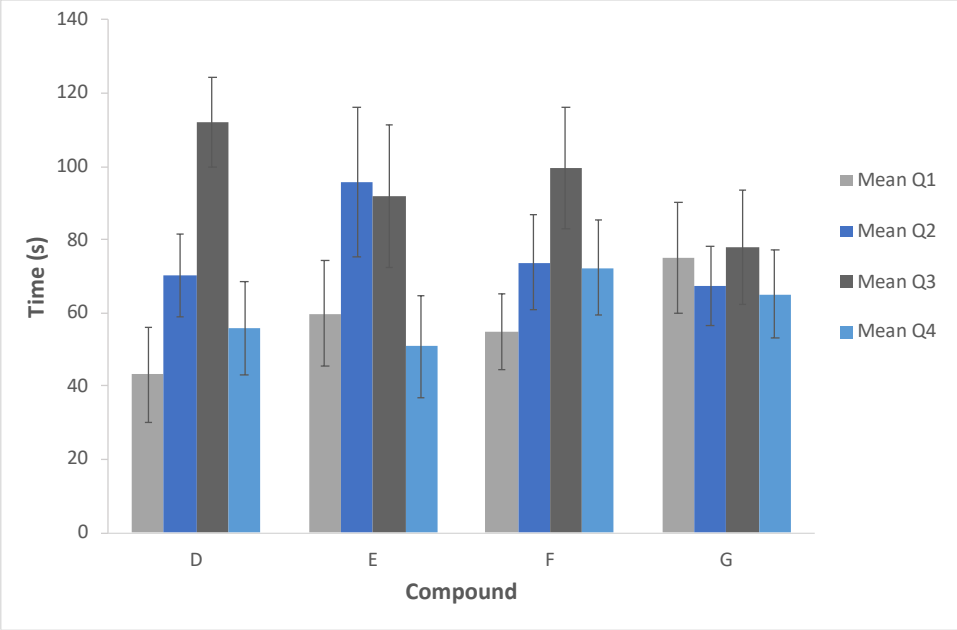

Figure C.2: Time copepodids spent in each arena quadrant. Vertical error bars represent standard error of the mean. Treatments: D = 200pptr isophorone; E = ASW; F = 200ppb isophorone; G = 200ppq isophorone. Quadrant 1 (Q1) is always the side in which the compound was added, whereas Q4 is the furthest from where the compound was added.

BIOGRAPHY OF THE AUTHOR

Kathryn "Katie" Liberman was born in Leonardtown, Maryland on February 14, 1995. She was raised in Sumner, Illinois and graduated as valedictorian from Red Hill High School in 2013. She attended the University of Maine and graduated *summa cum laude* in 2017 with a bachelor's degree in Marine Sciences. She remained in Maine for a gap year to work at the US Department of Agriculture and the Aquaculture Research Institute. She entered the Marine Biology graduate program at the University of Maine in the spring of 2019. After receiving her degree, Katie will try her hand at professional writing. Kathryn is a candidate for the Master of Science degree in Marine Biology from the University of Maine in December 2020.

Photo: Emily Tarr